INTERACTION OF PYRIDOXAL-5'-PHOSPHATE AND ACETOHYDROXY ACID ISOMEROREDUCTASE*

Eleanor M. Shematek** and Stuart M. Arfin

Department of Biochemistry
University of Pittsburgh School of Medicine
Pittsburgh, Pennsylvania 15213

Received May 17, 1971

SUMMARY: Acetohydroxy acid isomeroreductase is irreversibly inactivated upon binding approximately 8 moles of pyridoxal-5'-phosphate (PLP) followed by reduction with $NaBH_4$. Various analogs of PLP cause little or no inactivation. Inactivation is both more rapid and occurs at lower PLP concentrations in the presence of the specific cofactor NADPH, but the enzyme is not protected by substrates or products.

Pyridoxal-5'-phosphate (PLP) has been shown to interact with ϵ-amino groups of lysine residues in a number of enzymes to form Schiff base derivatives with concommitant reversible inactivation of enzymatic activity. Reduction of the Schiff base with $NaBH_4$ leads to irreversible inactivation. In the cases of phosphogluconate dehydrogenase (1) and fructose-1,6-diphosphate aldolase (2) it appears that the reactive lysine residues are located at the active site. The inactivation of pyruvate kinase (3), glutamate dehydrogenase (4) and hexokinase (5), while involving Schiff base formation between PLP and only a few of the lysine residues in these proteins is apparently not due to the formation of an active site-specific covalent linkage. Recently it has been shown that malate dehydrogenase is irreversibly inactivated by PLP even without reduction by $NaBH_4$ (6). It has been postulated that this inactivation is the result of an initial interaction between PLP and enzyme-bound lysine followed by formation of a thiazolidine-like complex with an enzyme-bound thiol.

* Supported by Grant 5 ROl AM 14102-02 from NIAMD.
** Pre-doctoral Trainee, NIH Grant 5 TOl GM 00149-12.

Acetohydroxy acid isomeroreductase (IR), an enzyme involved in branched chain amino acid biosynthesis, catalyzes an unusual reaction that probably proceeds via an initial acyloin-type rearrangement to an enzyme-bound α-keto-β-hydroxy acid intermediate followed by an NADPH-dependent reduction to the α, β-dihydroxy acid (7). As part of a study of structure-function relationships of IR we have examined the effect of PLP on the enzymatic activity. Here we present evidence that PLP binds to approximately 8 lysine residues of IR. Inactivation is both more rapid and occurs at lower PLP concentrations in the presence of the specific cofactor NADPH, but the enzyme is not protected by substrates or products.

METHODS: IR was purified and assayed as previously described (7). Treatment with PLP was carried out in 0.1M phosphate buffer, pH 7.6. Since reactivation to varying degrees occurred upon dilution into the assay solution, samples of the PLP treated enzyme were reduced by mixing with equal amounts of 10^{-3}M $NaBH_4$ for 15 minutes prior to assay. Spectra were recorded with a Beckman Spectrophotometer equipped with a Gilford recorder.

RESULTS AND DISCUSSION: Fig. 1A shows that treatment of IR with PLP followed by reduction with $NaBH_4$ leads to irreversible inactivation of the IR. Incubation of IR with NADPH, the specific cofactor, increases the rate of inactivation. Treatment of IR with $NaBH_4$ alone had no effect on the activity of the native enzyme. Fig. 1B shows that inactivation is achieved at considerably lower PLP concentrations in the presence of NADPH.

Table 1 shows the specificity for both PLP and pyridine nucleotide. It has previously been shown (7) that IR is specific for triphospyridine nucleotides in both the forward and reverse directions. Either NADP or NADPH, but not NAD or NADH, are required for the enzyme-catalyzed isomerization of α-keto-β-hydroxyisovalerate, the postulated intermediate, even though

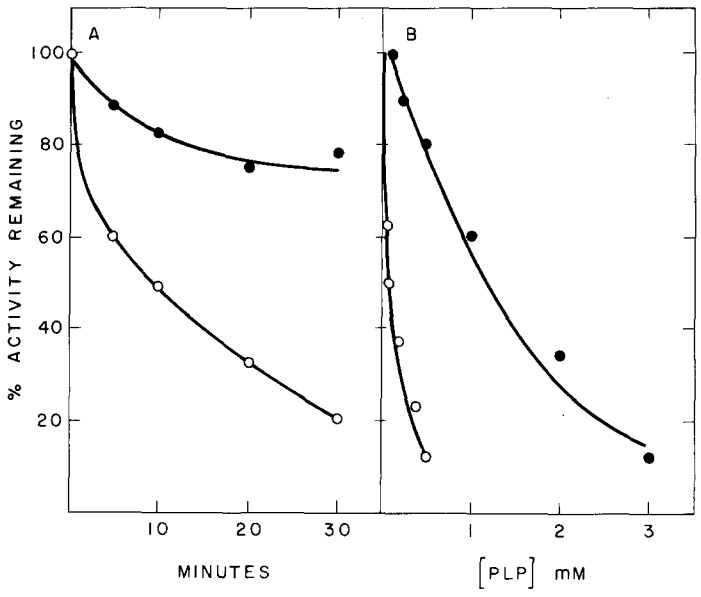

Fig. 1. A. Rate of inactivation of IR by PLP. Conditions: (●) 1.5 mμM IR and 0.5 mM PLP incubated as described in Methods. (o) Same plus 0.25 mM NADPH. B. Effect of PLP Concentration on Inactivation of IR. Conditions as in A, but incubation was for 30 minutes at the indicated PLP concentration. (●) No NADPH, (o) 0.25 mM NADPH.

---

no change in oxidation state occurs. It was postulated that binding of the triphosphopyridine nucleotides induced a conformational change in the enzyme needed for the binding of α-keto-β-hydroxyisovalerate. It seems probable that this same conformational change is responsible for the increased rate of inactivation by PLP. However, the failure to achieve significant protection against inactivation with products or substrates of the reaction as shown in Table 1 suggests that PLP does not bind at the acetohydroxy acid site.

Fig. 2 shows a difference spectrum of the reduced PLP-IR complex. The absorption maximum at 325 mμ is expected for the reduced ε-aminolysine-PLP complex. A ratio of 7.85 moles of inactivator bound per mole of enzyme was calculated from the molar extinction coefficient of 10,700

Table 1. Effect of Metabolites and PLP Analogs on Inactivation of IR

Additions to Preincubation	Pyridoxal Compound Added	% Activity Remaining
None	0.5 mM PLP	87%
2.5×10^{-4} M NADPH	0.5 mM PLP	17
2.5×10^{-4} M NADP	0.5 mM PLP	17
2.5×10^{-4} M NADH	0.5 mM PLP	67
2.5×10^{-4} M NAD	0.5 mM PLP	78
2.5×10^{-4} M NADPH + 2.5×10^{-2} M Dihydroxyisovalerate	0.5 mM PLP	20
2.5×10^{-4} M NADP + 2.5×10^{-2} M α-Acetolactate	0.5 mM PLP	20
2.5×10^{-4} M NADPH	0.5 mM Pyridoxal	82
2.5×10^{-4} M NADPH	0.5 mM Pyridoxamine	94
2.5×10^{-4} M NADPH	0.5 mM Pyridoxamine-5'-P	100

IR (0.066 mg) was preincubated for 15 min in 0.1 M phosphate, pH 7.6 + 10^{-2} M $MgCl_2$ with additions as noted in the table. PLP or analog was then added and incubated for 30 min. prior to reduction with $NaBH_4$ and assay. Final volume was 0.2 ml.

(8). Partially inactivated IR binds less inhibitor on a weight basis, but approximately the same amount on an active enzyme basis.

IR has a M.W. of 220,000, contains 124 lysine residues and apparently consists of 4 highly similar if not identical subunits (9). The stoichiometry

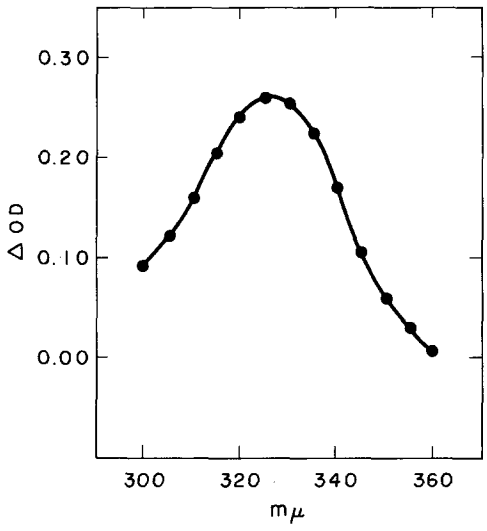

Fig. 2. Difference Spectrum of Reduced IR-PLP Complex. IR (0.83 mg/ml) was incubated with 0.5 mM PLP in the presence of 0.25 mM NADPH for 30 minutes at 25° and then reduced. The reference was a sample treated identically except that PLP was omitted.

---

of the reaction with PLP suggests that there may be 2 particularly reactive lysines per subunit, but their role, if any, in the catalytic process is unclear. These results do, however, suggest that for some enzymes, reactivity towards PLP may be a sensitive measure of ligand-induced conformational changes.

REFERENCES:

1. Rippa, M., L. Spanio and S. Pontremoli, Arch. Biochem. Biophys. 118, 48 (1967).
2. Shapiro, S., M. Enser, E. Pugh and B. L. Horecker, Arch. Biochem. Biophys. 128, 554 (1968).
3. Johnson, G. S. and W. C. Deal, Jr., J. Biol. Chem. 245, 238 (1970).
4. Anderson, B. M., C. D. Anderson and J. E. Churchich, Biochemistry 5, 2893 (1966).
5. Grillo, M. A., Enzymologia 34, 7 (1968).
6. Yost, F. J., Jr. and J. H. Harrison, Biochem. Biophys. Res. Comm. 42, 516 (1971).
7. Arfin, S. M. and H. E. Umbarger, J. Biol. Chem. 244, 1118 (1969).

8. Fischer, E.H., A.W. Forrey, J.L. Hedrick, R.C. Hughes, A.B. Kent and E.G. Krebs, in "Proceedings of the International Symposium on Chemical and Biological Aspects of Pyridoxal Catalysis" (E.E. Snell, P.M. Fasella, A. Braunstein, and A. Rossi-Fanelli, eds.) Pergamon, New York, 1963.
9. Shematek, E.M. and S.M. Arfin, in preparation.